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The results from this study have provided the first insights into how hyperthermophilic organisms obtain energy from the metabolism of C and N compounds, and how this can be achieved at high temperatures. These organisms have unusual pathways based on the rarely-used element tungsten (W), which is present in the novel enzymes AOR, FOR and GAPOR. W is seldom used in biological systems, the analogous element, molybdenum (Mo), is virtually ubiquitous. We propose that W is much more suited to catalyze low potential reactions (such as those catalyzed by GAPOR, AOR and FOR) at extreme temperatures, and that such reactions could not be catalyzed by Mo-containing enzymes. We have cloned and sequenced the first genes for any W-protein, and the first genes for hyperthermophilic oxidoreductases. In addition, a new pathway for peptide metabolism has been proposed, again involving new types of enzyme, such as VOR, IOR and ACS. We also provided the first definitive model for the evolution of mesophilic oxidoreductases from hyperthermophilic enzymes, and the first crystal structure for a hyperthermophilic enzyme (AOR) was obtained, providing the first insights in to protein stability at extreme temperatures.

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September 30, 1996

Dear Eric,

Please find attached two copies of my Final Progress Report for grant number N00014-90-J-1894 for the period June, 1993 - May, 1996.

I would like to thank both you and your office for supporting my research over this period. I feel we have been very productive and have made tremendous progress in understanding the metabolism and biochemistry of hyperthermophilic organisms.

Please do not hesitate to contact me if further information is required.

With best regards,

Yours sincerely,

Mwa

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FINAL PROGRESS REPORT

GRANT#: N00014-90-J-1894 R&T CODE: 3412-085

PRINCIPAL INVESTIGATOR: Michael W. W. Adams

INSTITUTION: University of Georgia

GRANT TITLE: "Metabolic and Enzymological Studies of Sulfur-Dependent Marine Hyperthermophiles"

AWARD PERIOD: 1 June 1993 - 31 May 1996

OBJECTIVES:

Hyperthermophiles are a recently discovered group of marine organisms that grow above 90°C. Virtually all are archaea. The majority are strictly anaerobic heterotrophs that utilize peptides and reduce elemental sulfur (S^0) to H_2S. Our aims are to understand the novel biochemistry that must be required to sustain life near 100°C.

ACCOMPLISHMENTS:

At the outset of this research we had already established that the growth of heterotrophic hyperthermophiles is dependent upon the rarely-used element, tungsten (W) and a novel tungstopterin-containing enzyme, aldehyde ferredoxin oxidoreductase (AOR), was proposed to play a key role in its primary metabolism. In the present award period, we cloned and sequenced the gene for AOR from *Pyrococcus furiosus* (Tmax 105°C, Pf) (1) and in collaboration with Dr. Doug Rees (Caltech), a crystal structure of AOR at 2.3Å resolution was obtained (2). This was the first structure of a hyperthermophilic, a W-, or a pterin-containing enzyme. Hence, it gave important insights into the nature and role of pterin cofactors as well as into potential mechanisms of protein stability (2). In addition, W was shown to affect the metabolic patterns of Pf (3). AOR was also been purified from *Thermococcus* ES-1 (Tmax 95°C, ES-1), an organism that is obligately dependent upon elemental sulfur (S^0) for growth (4). Moreover, from the related organism, *Thermococcus litoralis* (Tmax 98°C, Tl), a second type of W-containing aldehyde-oxidizing enzyme (FOR) was also purified (5), and its gene was cloned and sequenced (1). This enzyme had about 60% sequence similarity to Pf AOR showing that the two enzymes are closed related. We subsequently showed that, in addition to AOR and FOR, a third type of W-enzyme is present in these hyperthermophilic organisms. This was termed glyceraldehyde-3-phosphate oxidoreductase (GAPOR) and it was purified from *P. furiosus* (6). GAPOR was proposed to play a central role in a novel glycolytic pathway in these organisms (6) in which it replaces GAP dehydrogenase. Other glycolytic enzymes appear to be of the usual type. For example, we purified the enolase from Pf (7) and showed that it resembles analogous mesophilic enzymes except in thermal stability. In contrast to GAPOR, the other two tungstoenzymes, AOR and FOR, are proposed to function in peptide metabolism, as discussed below. However, we also showed that hyperthermophiles such as Pf are unique in that their growth is obligately dependent upon W (8). This cannot be replaced by

molybdenum, an analogous element that is utilized by virtually all other life forms (see 9, 10).

In the fermentation of peptides by the hyperthermophiles, we showed that the first step in the utilization of aromatic amino acids is catalyzed by transaminases (ATs). Both Tl and Pf were to contain two distinct types and these were purified (11,12). However, we also found that these organisms contain a new type of enzyme involved in the catabolism of aromatic amino acids which we term indolepyruvate oxidoreductase (IOR) (13). IOR produces aryl acetyl CoAs and CO_2 from the transaminated forms of the aromatic amino acids. Another key enzyme in peptide utilization, glutamate dehydrogenase (GDH), has also been purified from Tl (14) and the deep sea isolate ES-4 (15). Our investigations into the pathways of amino acid oxidation by these proteolytic hyperthermophiles also showed that in addition to IOR, they contain three types of 2-keto acid oxidoreductases and these have also been purified. While IOR is specific for aromatic keto acids (13) derived from the aromatic amino acids, another new enzyme which we term VOR uses keto acids derived from branched chain amino acids (16). The third enzyme is termed KGOR, and this is specific for 2-ketoglutarate (17). The fourth is POR, which mainly utilizes pyruvate, and this had already been purified from Pf in earlier studies. In the ONR-supported work, the genes for VOR and POR have been cloned and sequenced from *P. furiosus* and the POR genes have been sequenced from the bacterium *Thermotoga maritima* (Tmax 90°C: 18). From these data we have constructed an evolutionary model to show how the 2-keto acid oxidoreductases in mesophilic organisms arose from the gene fusion of ancestral genes now represented in the hyperthermophiles. Moreover, a unifying mechanism has been proposed to account for the diverse nature of these enzymes (10).

The fermentation of amino acids by these organisms is complicated by our finding that ES-1 expresses of an unusual alcohol dehydrogenase (ADH) which has been purified, and this reduces aldehydes to alcohols to dispose of excess reductant (19). Tl also contains a similar alcohol dehydrogenase (ADH) which was also purified (14). Both enzymes preferentially reduce aldehydes to alcohols. The question arose, therefore, as to the source of the aldehydes. We solved this by showing that POR, IOR and VOR not only oxidize 2-keto acids, they non-oxidatively decarboxylate them to the corresponding acid (20). Moreover, we also propose that the tungstoenzymes AOR and FOR have a similar role, that is, they function to oxidize such aldehydes to the corresponding acid (4, 10). The question also arose as the fate of the CoA-derivatives generated by POR, VOR, IOR and KGOR. We showed (21) that these are utilized by an enzyme known as acetyl CoA synthetase (ACS), which converts the CoA derivatives, ADP and phosphate to the acid and ATP. In fact, Pf has two ACS isoenzymes, wherein ACS I utilizes the products of the POR and VOR reactions, whereas only ACS II utilizes the products of the IOR reaction. Neither enzyme uses succinyl CoA, which is produced by KGOR (21). Hence, we have proposed a completely new pathway by which amino acids are converted to carboxylic acids with energy conservation by ACS (10).

We have also shown that S° reduction by hyperthermophiles such as Pf is an energy yielding reaction in Pf, in spite of

previous assumptions (3), and two S°-reducing enzymes have been characterized: sulfhydrogenase (22) and a NADP-dependent sulfide dehydrogenase (SuDH) (23). We also found that SuDH functions as a ferredoxin:NADP oxidoreductase. This led to the elucidation of the pathway of electron transfer with the reconstitution of a system that would evolve H_2 from pyruvate at 90°C. This comprised pyruvate oxidoreductase, ferredoxin, SuDH, NADP and hydrogenase (24). Hence, this shows how all of the reduced ferredoxin generated during the fermentation of both peptides and carbohydrates can be oxidized to produce H_2.

Finally, we also initiated a project to map by 2D-electrophoresis the proteins of *P. furiosus* (25) which will hopefully be of use when results from the genomic sequencing project of *P. furiosus* become available. In addition to refs. 9, 10, other aspects of the research presented here are summarized in the reviews given in refs. 26 - 31.

SIGNIFICANCE:

Our results have provided the first insights into how hyperthermophilic organisms obtain energy from the metabolism of C and N compounds, and how this can be achieved at high temperatures. These organisms have unusual pathways based on the rarely-used element tungsten (W), which is present in the novel enzymes AOR, FOR and GAPOR. W is seldom used in biological systems, the analogous element, molybdenum (Mo), is virtually ubiquitous. We propose that W is much more suited to catalyze low potential reactions (such as those catalyzed by GAPOR, AOR and FOR) at extreme temperatures, and that such reactions could not be catalyzed by Mo-containing enzymes (12). We have cloned and sequenced the first genes for any W-protein, and the first genes for hyperthermophilic oxidoreductases. In addition, a new pathway for peptide metabolism has been proposed, again involving new types of enzyme, such as VOR, IOR and ACS. We also provided the first definitive model for the evolution of mesophilic oxidoreductases from hyperthermophilic enzymes, and the first crystal structure for a hyperthermophilic enzyme (AOR) was obtained, providing the first insights in to protein stability at extreme temperatures.

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OBJECTIVES

Hyperthermophilic Microorganisms Grow Near and Above 100°C:

- how do they stabilize proteins and cofactors at 100°C ?

- do they utilize conventional or novel primary metabolic pathways ?

- do they use conventional mechanisms for energy conservation ?

ACCOMPLISHMENTS

- discovered that the growth of both carbohydrate- and peptide-fermenting "hyperthermophiles" is dependent upon tungsten (W), an element seldom used in biology.

- obtained the crystallographic structure of a novel tungstopterin-containing enzyme (AOR) at 2.3 Å resolution, the first for a W-, a pterin- or a hyperthermophilic enzyme.

- purified a third W-enzyme (GAPOR) which is involved in carbohydrate metabolism. This is in addition to AOR and FOR, which are involved in peptide catabolism.

- cloned and sequenced the first genes for any W-protein (AOR and FOR), and the first genes for any hyperthermophilic oxidoreductases.

- obtained the first definitive model for the evolution of mesophilic oxidoreductases from hyperthermophilic enzymes

- purified and characterized two more novel enzymes involved in peptide catabolism: ketoisovalerate oxidoreductase (VOR) and an unusual alcohol dehydrogenase.

- purified and characterized sulfide dehydrogenase, a novel enzyme involved in the reduction of elemental sulfur (S°), and defined the pathway for H_2 production from pyruvate.

SIGNIFICANCE

- tungsten (W) is a key element in the primary metabolism of hyperthermophiles and first identification of a life form (the heterotrophic hyperthermophiles) that is obligately dependent upon W for growth

- first rationalization of W (but not the ubiquitous element, Mo) is essential for life near and above 100°C.

- several novel enzymes (including AOR, FOR, GAPOR, VOR, IOR, FNOR, ACS) have been discovered, purified and characterized

- proposed a new pathway of peptide fermentation and a modified glycolytic route

- first information on the mechanism of sulfur reduction by hyperthermophiles and the first characterization of enzymes that reduce S° at extreme temperatures.

- first definitive model for the evolution of mesophilic enzymes from enzymes of hyperthermophiles.

- first crystal structure of a hyperthermophilic or a W-containing enzyme.

M. W. W. Adams, U. of Georgia, Sept. 1996